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Dehalococcoides mccartyi Strain DCMB5 Respires a Broad Spectrum of Chlorinated Aromatic Compounds

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Polyhalogenated aromatic compounds are harmful environmental contaminants and tend to persist in anoxic soils and sediments. *Dehalococcoides mccartyi* strain DCMB5, a strain originating from dioxin-polluted river sediment, was examined for its capacity to dehalogenate diverse chloroaromatic compounds. Strain DCMB5 used hexachlorobenzenes, pentachlorobenzenes, all three tetrachlorobenzenes, and 1,2,3-trichlorobenzene as well as 1,2,3,4-tetra- and 1,2,4-trichlorodibenzo-*p*-dioxin as electron acceptors for organohalide respiration. In addition, 1,2,3-trichlorodibenzo-*p*-dioxin and 1,3-, 1,2-, and 1,4-dichlorodibenzo-*p*-dioxin were dechlorinated, the latter to the nonchlorinated congener with a remarkably short lag phase of 1 to 4 days following transfer. Strain DCMB5 also dechlorinated pentachlorophenol and almost all tetra- and trichlorophenols. Tetrachloroethene was dechlorinated to trichloroethene and served as an electron acceptor for growth. To relate selected dechlorination activities to the expression of specific reductive dehalogenase genes, the proteomes of 1,2,3-trichlorobenzene-, pentachlorobenzene-, and tetrachloroethene-dechlorinating cultures were analyzed. Dcmb_86, an ortholog of the chlorobenzene reductive dehalogenase CbrA, was the most abundant reductive dehalogenase during growth with each electron acceptor, suggesting its pivotal role in organohalide respiration of strain DCMB5. Dcmb_1041 was specifically induced, however, by both chlorobenzenes, whereas 3 putative reductive dehalogenases, Dcmb_1434, Dcmb_1339, and Dcmb_1383, were detected only in tetrachloroethene-grown cells. The proteomes also harbored a type IV pilus protein and the components for its assembly, disassembly, and secretion. In addition, transmission electron microscopy of DCMB5 revealed an irregular mode of cell division as well as the presence of pili, indicating that pilus formation is a feature of *D. mccartyi* during organohalide respiration.

Polyhalogenated aromatics comprise several classes of compounds that are notorious as environmental pollutants, which include polychlorinated dibenzo-*p*-dioxins (PCDD) and -furans (PCDF), polychlorinated biphenyls (PCB), polybrominated diphenyl ethers (PBDE), halogenated benzenes, and phenols. Although current emissions of these compounds have become strongly reduced, a high burden of these pollutants still remains, often enriched at former chlorine-based industrial sites (1, 2). The anoxic sediments of the Spittelwasser Creek in the Bitterfeld-Wolfen district in central Germany constitute such a hot spot that is highly polluted by PCDDs, PCDFs, di- to hexachlorobenzenes, chlorinated phenols, hexachlorocyclohexane, and many other halogenated aromatic and aliphatic organic compounds (3).

The intrinsic bioremediation capacity of these sediments was demonstrated by anoxic microcosms reductively dechlorinating spiked chlorinated dibenzo-*p*-dioxins (4, 5). *Dehalococcoides mccartyi* strain DCMB5 was enriched from these microcosms and was isolated initially using 1,2,3,4-tetrachlorodibenzo-*p*-dioxin (TeCDD) or 1,2,4-trichlorodibenzo-*p*-dioxin (TrCDD) and subsequently 1,2,3-trichlorobenzene (TCB) as electron acceptors, hydrogen as the electron donor, and acetate and CO_2 as carbon sources (6). Recently, the genome of strain DCMB5 was sequenced and revealed the presence of 23 *rdhAB* genes encoding the catalytic subunit A and the putative membrane anchor subunit B, respectively, of reductive dehalogenases (or reductive dehalogenase homologous proteins, Rdh proteins, if not yet biochemically characterized) (7). This finding suggests that the bacterium has a high capacity for organohalide respiration with a broad range of pollutants (8).

To explore the potential of strain DCMB5 to contribute to the natural attenuation of the contaminants in the polluted sediments, the range of organohalides that can be reductively dehalogenated and serve as electron acceptors for growth was investigated. Recently, quantitative proteome analysis has become significantly more sensitive and thus provides high coverage of the proteome of cultures that grow only to limited biomass density, as is the case with *D. mccartyi* strains (9–11). Here, a shotgun proteome analysis (10) was used to compare Rdh formation during growth on three different organohalides. In addition, we report

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the morphogenesis of strain DCMB5 and demonstrate pilus formation.

MATERIALS AND METHODS

Chemicals. Chlorinated benzenes (99% purity) and the isomers of hexachlorocyclohexane were purchased from Sigma-Aldrich (Steinheim, Germany). The chlorinated dibenzo-*p*-dioxins used in this study were purchased from AccuStandard Inc. (New Haven, CT), and the chlorophenols were from Sigma-Aldrich, AppliChem (Darmstadt, Germany), Serva (Heidelberg, Germany), and Merck (Darmstadt, Germany). Tetrachloroethene (PCE), trichloroethene (TCE), and *cis*- and *trans*-dichloroethene (DCE) were obtained from Merck (Darmstadt, Germany), and vinyl chloride (VC) was bought from Linde Gas AG (Pullach, Germany). All chemicals were of the highest quality available.

Growth medium and cultivation conditions. Stock cultures of strain DCMB5 were maintained in 30- to 60-ml two-liquid-phase cultures (6) in anoxic, Ti(III)-citrate (1 mM)-reduced, bicarbonate-buffered, purely synthetic mineral medium (12). Cultures were overlaid with 0.05 volume of either 200 or 400 mM 1,2,3-TCB dissolved in hexadecane, resulting in a nominal concentration of 10 and 20 mM, respectively. Hydrogen served as the electron donor and was added to the headspace to give an overpressure of 50,000 Pa (7.3 lb/in^2). Acetate (5 mM) was added as a carbon source. The cultures were incubated statically at 30°C in the dark and served as starting material (10%, vol/vol) for the substrate specificity experiments described below.

Dechlorination of different tri- to hexachlorinated benzenes was studied in replicate 30-ml glass bottles filled with 20 ml of medium and 10% (vol/vol) inoculum. TCBs and 1,2,3,4- and 1,2,3,5-tetrachlorobenzenes (TeCBs) were added from 500 mM stock solutions in acetone directly to the medium, resulting in a nominal concentration of 50 μM. 1,2,4,5-TeCB, hexachlorobenzene (HCB), and pentachlorobenzene (PeCB) are less soluble in acetone, and therefore, they were added from 300 μM stock solutions to empty serum bottles and the acetone was evaporated prior to the addition of anoxic medium. The reported aqueous solubility of chlorinated benzenes ranges from 100 to 250 μM for TCBs to 18 nM for HCB (13). For growth experiments, 1,2,4,5-TeCB, PeCB, and HCB were added as crystals (~5 mg) to each bottle to provide a continuous dissolution of the electron acceptor. The more water-soluble TCBs and 1,2,3,4- and 1,2,3,5-TeCBs were repeatedly added in 50 μM doses from acetone stocks to the anaerobic cultures. Duplicate, noninoculated cultures supplemented with the tested chlorobenzenes served as abiotic controls.

For the study of the conversion of chlorinated dibenzo-*p*-dioxins, a miniaturized culture set-up using 1.5-ml culture volumes in 20-ml crimp-sealed headspace vials was used to reduce the required amount of the hazardous and expensive dioxin congeners and to allow the combined dioxin measurement in the gas phase and culture fluid using standardized solid-phase microextraction (SPME) and fluid-fluid extraction protocols, respectively, as previously described (5). Growth experiments were conducted in 10-ml culture volumes. Respective congeners were added from stock solutions (300 μM to 1.3 mM) in acetone to sterile vials to give a nominal concentration of 25 μM or 250 μM. Acetone was vaporized by use of a continuous, sterile nitrogen flow. The aqueous solubility of chlorinated dioxins ranges from 1 to 2 μM for monochlorodibenzo-*p*-dioxin (MCDD) to 20 nM for 1,2,3,4-TeCDD (14). Further preparation and cultivation (30°C) were performed in a Coy glove box (Coy, Grass Lake, Michigan) (atmosphere, 5% H_2 and 95% N_2). An appropriate volume of anoxic medium was inoculated using 10% (vol/vol) of a preculture and dispensed in 1.5-ml aliquots into the headspace vials. The vials were subsequently closed with Teflon-coated butyl rubber stoppers and aluminum crimps. A set of cultures without inoculum served as controls. Triplicate cultures were harvested and analyzed at each sampling point.

Dechlorination of trichlorophenol (TCP), tetrachlorophenol (TeCP), and pentachlorophenol (PCP) was examined in duplicate or triplicate 30-ml glass bottles filled with 20 ml anoxic medium. Chlorophenols were spiked from anoxic stock solutions (5 mM) dissolved in sodium hydroxide (5 mM) to final concentrations of 50 μM (TCP and PCP) and 30 μM (TeCP).

Reductive dechlorination of the chlorinated ethenes PCE, TCE, *cis*- and *trans*-DCE, and VC was investigated in 120-ml glass bottles containing 60 ml of anoxic medium. For each compound, triplicates and two chemical controls were prepared. Chlorinated ethenes were added from neat substances yielding, per bottle, 23.5 (PCE), 27 (TCE), and 31 (*cis*- and *trans*-DCE) μmol. Using a gas-tight syringe, 32 μmol of VC was added. Cultures were inverted for incubations.

Hexachlorocyclohexane (HCH) conversion (α-, β-, γ-, and δ-HCH; 10 or 20 μM) was investigated in sodium sulfide (1 mM)-reduced medium (22.5 ml in 50-ml glass bottles) (15) due to the abiotic dechlorination of HCH in the presence of Ti(III) citrate (16). Triplicate cultures incubated with 50 μM 1,2,3-TCB served as a positive control. All experiments were performed at least twice.

Microscopy, cell counting, and growth. Growth of *Dehalococcoides* was monitored by microscopic counting of 4′,6-diaminido-2-phenyl-indole (DAPI) (2.5 μg ml^{-1})-stained cells immobilized in agarose (17) using an Axioskop equipped with an AxioCam Mrc digital camera and AxioVision software (v. 4.6.3) (Zeiss, Oberkochen, Germany). The cell diameter was determined from phase-contrast images.

Electron microscopy. Cells were gently concentrated from 1,2,3-TCB-grown cells (2-ml volumes) by centrifugation for 45 min at 600 × *g* and room temperature. The supernatant was carefully removed except for a residual 50 μl. The cells were resuspended in the remaining volume and 3 μl of the suspension were immediately transferred to Formvar-coated copper grids. After negative staining with 2% (wt/vol) aqueous uranyl acetate, samples were observed with an EM 900 transmission electron microscope (Carl Zeiss Microscopy, Oberkochen, Germany) at an acceleration voltage of 80 kV. Electron micrographs were taken with a slow-scan camera (Variospeed slow-scan charge-coupled device [SSCCD] camera; SM-1k-120; TRS, Moorenweis, Germany).

Analytical techniques. Dechlorination of chlorinated benzenes in the two-liquid phase cultures was monitored by the coulometric measurement of the released chloride using a Chlor-o-Counter (Flohr Instrumenten, The Netherlands), or, for the analysis of the substrate spectrum, chlorobenzenes were extracted from the water phase with *n*-hexane (1:1 ratio) by sonication in an ultrasonic bath (35 kHz, 15 min) followed by inverted shaking at 160 rpm at room temperature overnight. Compounds were quantified using a Shimadzu gas chromatograph with a flame ionization detector (GC-FID) and an Rtx35 capillary column and 1,3,5-tribromobenzene was used as an internal standard. Further details of the GC analysis are provided in the supplemental material.

Monochlorinated and the nonchlorinated dibenzo-*p*-dioxin were analyzed from the headspace of the cultures by SPME (100-μm polydimethylsiloxane coated fibers; Supelco). Samples were preconditioned for 2 h at 80°C, and the fiber was equilibrated 35 min at 80°C and desorbed for 195 s (injection port, 260°C), followed by a splitless injection (0.7 min). The compounds were separated on a DB-608 capillary column and analyzed by GC-FID as described in the supplemental material. Subsequently, the liquid cultures were extracted using *n*-hexane (three times with 1 ml each time) and the di- to tetrachlorinated congeners were analyzed on the Rtx35 capillary column (see above) using the GC-FID and the temperature program described previously (18) with 2,4,8-trichlorodibenzofuran dissolved in heptamethylnonane as the internal standard.

Chlorophenols were monitored by either high-performance liquid chromatography (HPLC) or GC with electron capture detection (GC-ECD). For HPLC analysis (Elite LaChrom; VWR, Darmstadt, Germany) a C_{18} column (length, 250 mm; inside diameter [i.d.], 4 mm; bead diameter, 5 μm; LiChrospher) was used with methanol-water-acetic acid (65:35:0.1, vol/vol/vol) as the solvent, as described in reference 17. Alternatively, the Shimadzu GC-ECD was used for the analysis of the dechlorination of PCP and 2,3,4,5-TeCP and of 2,4- and 3,4-dichlorophenol (DCP), which were not separated by HPLC (see the supplemental material).

To detect chlorinated ethenes, 0.5-ml headspace samples were taken

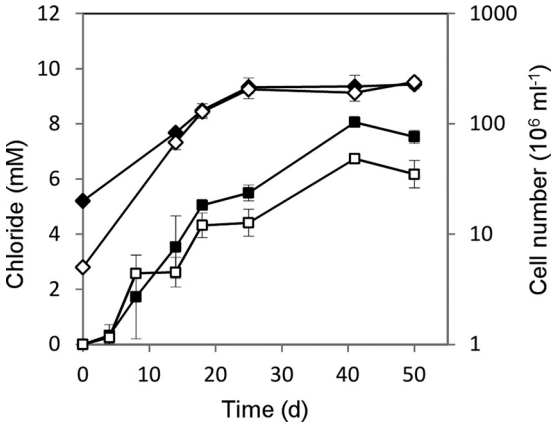

FIG 1 Growth of strain DCMB5 (diamonds) and release of chloride (squares) from 1,2,3-TCB. 1,2,3-TCB was supplied by overlaying a 400 mM solution in hexadecane, resulting in a nominal concentration of 20 mM. Cultures were inoculated with 5×10^6 cells ml^{-1} (open symbols) or 2×10^7 cells ml^{-1} (closed symbols). Mean values and standard deviations from duplicate cultures are shown.

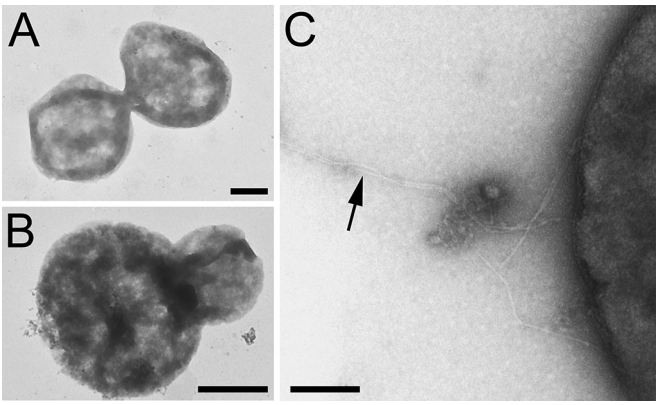

FIG 2 Transmission electron micrographs of uranylacetate-stained cells harvested from the exponential growth phase. Pairs of cells with equal (A) or unequal (B) sizes suggest an irregular mode of cell division. (C) Cell-attached pili. The subfilaments (arrows) have a diameter of 3 nm. Bars, 0.5 μm (A and B) and 0.1 μm (C).

and analyzed as described previously (19, 20) using gas chromatography (Chrompack CP-3800; Varian, Middelburg, The Netherlands) with an FID. The temperature program used was as follows: 1 min at 100°C, 50°C per min to 225°C, held for 2.5 min. The FID was operated at 250°C, and helium was used as the carrier gas (0.69×10^5 Pa; 11.5 ml per min).

Proteome analysis. Samples containing 2×10^9 cells were taken from triplicate cultures grown on PeCB, TCB, and PCE and filtered through a sterile polyvinylidene fluoride filter (pore size, 0.22 μm; Roth, Karlsruhe, Germany). After the retained cells had been flushed off the filter, they were pelleted by centrifuging for 1 h with 4,000 × *g* at 16°C. Pellets were stored at −20°C until further processing.

Cell lysis was carried out by three cycles of freeze (liquid nitrogen)-thawing, and the cell lysate was reduced (50 mM dithiothreitol with shaking for 1 h at 30°C) and alkylated (130 mM iodoacetamide with shaking for 1 h in the dark). The cell lysate was fractionated by centrifugation for 1 h with 100,000 × *g* at 4°C, and both fractions, the pellet and the supernatant, were analyzed in parallel. To maximize the yield of detectable proteins from the small amount of available biomass, the pellet was not subjected to washing steps and was therefore expected to contain membrane proteins and soluble cytoplasmic proteins. Proteins were digested overnight at 37°C using sequencing-grade trypsin (Promega, Madison, WI). Proteolysis was stopped by adding formic acid to a final concentration of 1% (vol/vol). Tryptic peptides were analyzed using a nanoflow uHPLC system coupled to an Orbitrap mass spectrometer (Thermo Scientific) (for details, see the supplemental material).

The acquired LC-MS data were analyzed using two search engines, MS Amanda and Sequest HT, and compared against the database of *D. mccartyi* DCMB5 (details of the analysis are given in the supplemental material). Peptides were considered identified with a false discovery rate (FDR) of <1%. Proteins with ≥1 high-confidence peptides were regarded as suitable for further analysis (see Table S1 in the supplemental material). Proteins detected by characteristic mass fragments in at least one of three biological replicates were considered identified. Those occurring in at least two replicates of a corresponding fraction were used for quantification by calculating the area under the peptide peaks. Protein areas were $\log_{10}$ transformed and median normalized. The relative abundance of selected proteins was compared to the housekeeping protein GroEL (Dcmb_1287). Subcellular localization of proteins was retrieved from the PSORTb database (21).

RESULTS

Morphology and growth of strain DCMB5 during organohalide respiration with 1,2,3-trichlorobenzene. Growth of strain DCMB5 was monitored in two-liquid-phase cultures with 1,2,3-TCB inoculated with 5×10^6 or 2×10^7 cells ml^{-1} (Fig. 1). Dechlorination occurred until day 42, releasing up to 8 mM chloride. The cells grew exponentially until day 18 and stopped growing after 25 days, when the cell number had reached 2×10^8 cells ml^{-1}; cessation of growth was independent of the size of the initial inoculum. The growth yield was 4.6×10^7 ($\pm 0.1 \times 10^7$) and 3.6×10^7 ($\pm 1 \times 10^7$) cells μmol^{-1} of chloride released for the cultures inoculated with 5×10^6 or 2×10^7 cells ml^{-1}, respectively. The diameter of the flat cells was measured in the cultures using phase-contrast microscopy and indicated a highly variable cell size during exponential growth. Figure S1 in the supplemental material documents that the majority of cells (>50%) inoculated from a stationary-phase culture into fresh medium had a diameter between 1 and 1.4 μm and only 10% of the cells had a diameter below 0.8 μm. During exponential growth, the fraction of small cells increased ~6-fold, showing a broad range of different-sized cells between 0.55 μm and 1 μm. Samples were taken from all cultures after 4, 8, 14, and 18 days for transmission electron microscopy to study the morphology in the phase of intensive cell division. At days 14 and 18, many cells appeared as pairs. The pairs consisted of equal-sized and unequal-sized cells, often with a bud-like appearance (Fig. 2), suggesting an asymmetric mode of cell division. Interestingly, at days 14 and 18, several cells in both cultures inoculated at 5×10^6 cells ml^{-1} exhibited pilus-like structures, which often appeared in pairs with an individual diameter of 3 nm (Fig. 2). They were not detected after 4 and 8 days of incubation, nor were they observed in stationary-phase cells or in the cultures inoculated with 2×10^7 cells ml^{-1}.

Spectrum of chlorinated benzenes used for organohalide respiration. Reductive dechlorination of all isomers of tri- and tetrachlorobenzene, PeCB, and HCB was analyzed at final concentrations of 50 μM. All isomers except 1,3,5-TCB were reductively dechlorinated to less-chlorinated products. 1,2,4-TCB was degraded to only small amounts (1 μM) of 1,4-dichlorobenzene (DCB). HCB and PeCB were both dechlorinated to 1,2,4-TCB and

TABLE 1 Final dechlorination products and growth of strain DCMB5 when cultivated on different chlorinated benzenes

Electron acceptor	Concn of final dechlorination product (μM)[a]				Increase in cell no.[b]
	1,3,5-TCB	1,2,4-TCB	1,4-DCB	1,3-DCB	
1,2,3-TCB				55.8 ± 4.9	0.7×10^7
1,2,3,5-TeCB	28.5 ± 3.9				0.1×10^7
1,2,3,4-TeCB		41.5 ± 6.6	1.1 ± 0.1		0.5×10^7
1,2,4,5-TeCB		67.7 ± 11.0	13.6 ± 2.4		0.3×10^7
PeCB	21.3 ± 1.8	29.1 ± 0.5	2.3 ± 0.4		1.2×10^7
HCB	14.7 ± 2.0	22.3 ± 3.8	3.5 ± 0.5		1.0×10^7

[a] Values are means and SD for duplicate samples, determined after 27 days of incubation.
[b] Difference in cell numbers obtained at the beginning and after 27 days.

1,3,5-TCB. The dechlorination product of 1,2,3,4- and 1,2,4,5-TeCB was 1,2,4-TCB, and 1,2,3,5-TeCB was dechlorinated to 1,3,5-TCB. In the abiotic controls, no dechlorination products were formed, although a decrease of the spiked TCBs was observed, suggesting physical losses by sorption and/or evaporation. All cultures except the cultures incubated with 1,3,5- and 1,2,4-TCB were transferred into fresh medium (10%, vol/vol). The same dechlorination products were formed, indicating that the dechlorination was independent of residual 1,2,3-TCB, which might have been transferred from the preculture. To test if these compounds sustained growth by organohalide respiration, they were applied at higher concentrations: HCB, PeCB, and 1,2,4,5-TeCB were applied as crystals to the medium, and 1,2,3-TCB, 1,2,3,4-TeCB, and 1,2,3,5-TeCB were repeatedly fed from acetone stock solutions each to a final concentration of 50 μM at the beginning of cultivation and again after 11, 25, and 53 days. Again, the same products were formed as before; however, in cultures producing 1,2,4-TCB, a distinct formation of 1,4-DCB was observed (Table 1). No further increase in dechlorination products was observed after 27 days; the reductive dechlorination of PeCB is shown as an example (Fig. 3). This might reflect a decreased bioavailability of the electron acceptor and physical losses of the products formed. The increase in cell number at day 27 is given in Table 1 and indicated that strain DCMB5 can utilize 1,2,3-TCB, all tetrachlorinated benzenes, PeCB, and HCB as electron acceptors for growth.

Dechlorination of chlorinated dibenzo-*p*-dioxins and growth. To test the spectrum of chlorinated dibenzo-*p*-dioxins utilized, strain DCMB5 was incubated with 25 μM 1,2,3,4-TeCDD, 1,2,3-TrCDD, 1,2,4-TrCDD, 1,3-dichlorodibenzo-*p*-dioxin (DCDD), or 2,3-DCDD. Within 1 to 4 days, dechlorination products were formed from 1,2,3,4-TeCDD, 1,2,4-TrCDD, 1,2,3-TrCDD, and 1,3-DCDD, whereas 2,3-DCDD was not dechlorinated even after 8 days (see Table S2 in the supplemental material). To test growth of strain DCMB5 with 1,2,3,4-TeCDD and 1,2,4-TrCDD, two subsequent 10% (vol/vol) transfers were established in duplicate on the respective dioxin congener. In the second transfer, 1,2,3,4-TeCDD was transformed within 27 days to 34 (±4) μM 2,3-DCDD, 3.7 (±0.3) μM 2-MCDD, and 0.1 (±0.07) μM 1,3-DCDD, respectively. 1,2,4-TrCDD was transformed to 101 (±1.3) μM 1,3-DCDD and 15 (±1.2) μM 2-MCDD. The cells were counted at the beginning and after 109 days of incubation. The cell numbers increased from 3.4×10^6 to

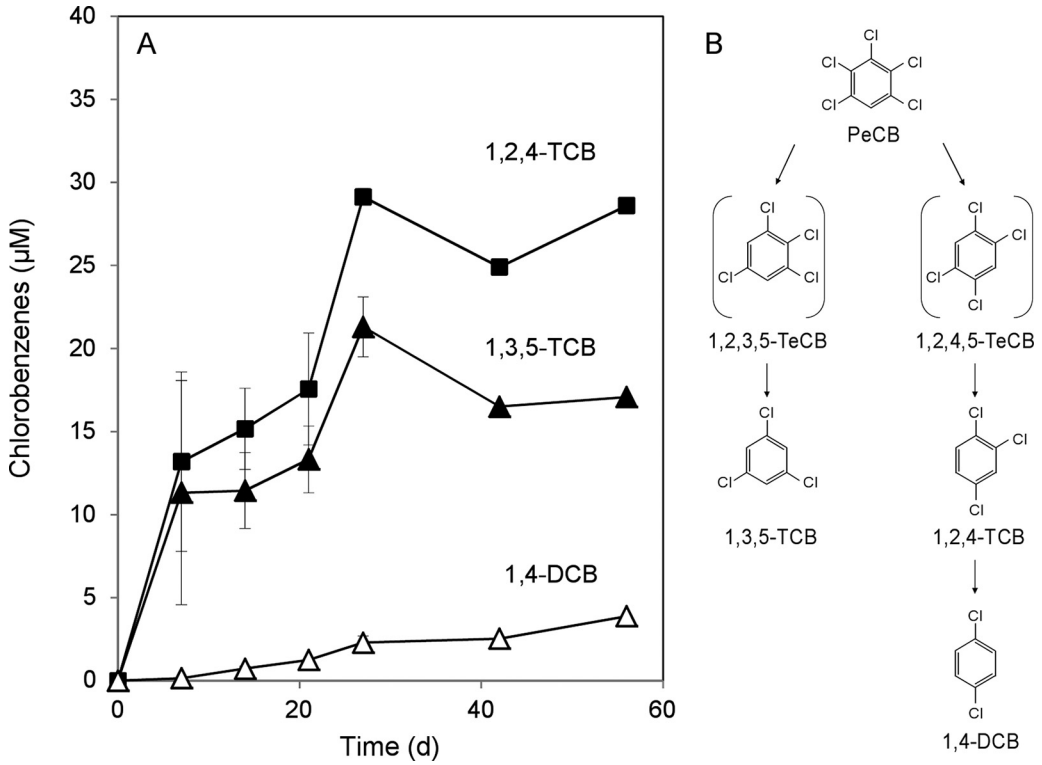

FIG 3 (A) Reductive dechlorination of PeCB to 1,2,4-TCB, 1,3,5-TCB, and 1,4-DCB. PeCB was added in crystalline form to the medium and was not quantified. Mean values and SD from duplicate cultures are shown. (B) Proposed dechlorination pathway of PeCB.

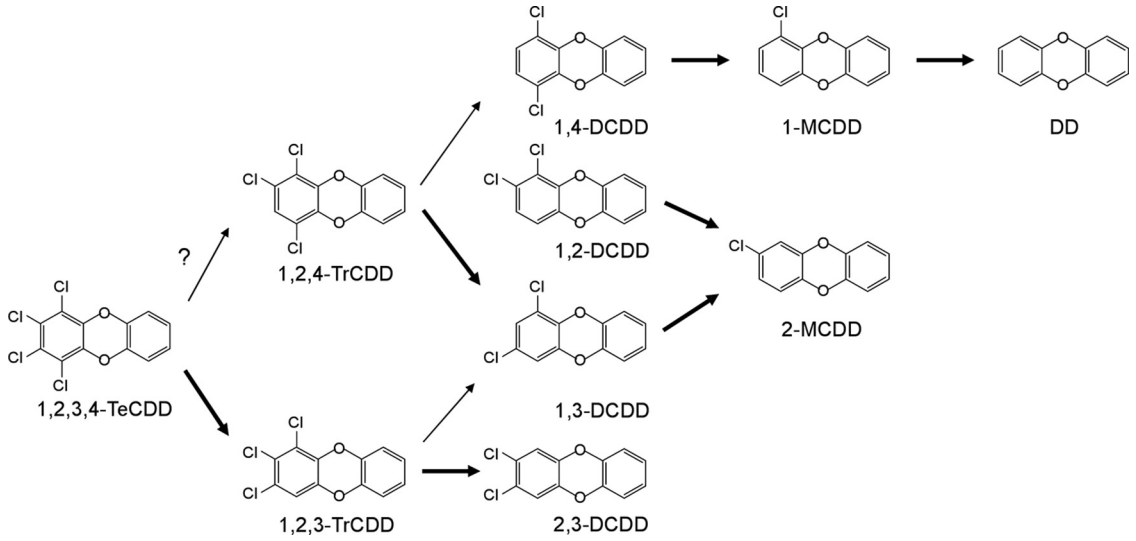

FIG 4 Observed dechlorination pathways for 1,2,3,4-TeCDD and lower chlorinated dibenzo-*p*-dioxins in strain DCMB5. Major and minor dechlorination reactions are indicated by thick and thin arrows, respectively. The question mark indicates that the reaction has not yet been verified.

37.5 ($\pm$1.4) $\times$ 10^6 cells ml^{-1} and from 2.6 $\times$ 10^6 to 30.8 ($\pm$7) $\times$ 10^6 cells ml^{-1} (values are means $\pm$ standard deviations [SD] from duplicate cultures) during cultivation with 1,2,3,4-TeCDD and 1,2,4-TrCDD, respectively. The 10-fold increase of cell numbers indicated growth of strain DCMB5 at the expense of 1,2,3,4-TeCDD and 1,2,4-TrCDD dechlorination. Interestingly, in the first transfer with 1,2,4-TrCDD, trace amounts of 1-MCDD and of nonchlorinated dibenzo-*p*-dioxin (DD) were observed. As the reductive dechlorination of 1,3-DCDD did not yield 1-MCDD (see Table S2 in the supplemental material), another DCDD congener must be the precursor. The reductive dechlorination of 1,2- and 1,4-DCDD as further potential intermediates of 1,2,4-TrCDD dechlorination was analyzed. While 1,2-DCDD was dechlorinated to 2-MCDD, 1,4-DCDD was dechlorinated to 1-MCDD and DD (see Table S2 in the supplemental material). Subsequently, reductive dechlorination of both MCDDs was tested and revealed formation of DD only from 1 MCDD (see Table S2 in the supplemental material). The observed pathways of reductive dechlorination of dibenzo-*p*-dioxins are summarized in Fig. 4.

Dechlorination of chlorinated phenols. Reductive dechlorination of PCP, all three TeCPs, all six TCPs, and 2,3-DCP was investigated. With the exception of 2,3-DCP, all tested chlorophenols were reductively dechlorinated. PCP was dechlorinated via the transient formation of all three TeCPs (in the order of decreasing amounts: 2,3,4,6-TeCP > 2,3,5,6-TeCP > 2,3,4,5-TeCP) to the final products 2,4,6-, 2,4,5-, and 2,3,5-TCP, 3,5-DCP, and 2,4- or 2,5-DCP (see Fig. S2 in the supplemental material). Based on the results obtained from separate incubations with the individual chlorophenols (Table 2), we propose the PCP dechlorination pathway shown in Fig. S3 in the supplemental material. Further dechlorination of the final dichlorophenol products was not observed. In addition, the dechlorination of 2,3,4-, 2,3,6-, and 3,4,5-TCP, which are not formed during PCP transformation, was analyzed (Table 2). Dechlorination of most chlorophenols added to a concentration of 30 to 50 μM was complete within 10 to 25 days, with the exception of that of 2,4,5-TCP, which was dechlorinated to only traces of 2,4- and 2,5-DCP. The fastest dechlorination was observed for 2,3,4-TCP and 3,4,5-TCP, which were completely transformed within 6 days.

Growth with 2,3,6-TCP as the electron acceptor was tested after a second transfer on this chlorophenol. The increase in the cell number from 4.6 $\times$ 10^6 ml^{-1} to 10.4 $\times$ 10^6 ($\pm$1.2 $\times$ 10^6) ml^{-1} during complete dechlorination of two 50 μM doses of 2,3,6-TCP indicated growth on 2,3,6-TCP.

Reductive dechlorination of chlorinated aliphatic compounds. The reductive dechlorination of PCE, TCE, *cis*- and *trans*-DCE, and VC was studied in triplicate cultures. No dechlorination products were formed over a period of 82 days from TCE, the two DCE isomers, and VC, whereas the initially fed PCE (23.5 μmol per bottle) was dechlorinated within 15 days to 80 mol% TCE. Three further additions of PCE were rapidly dechlorinated

TABLE 2 Reductive dechlorination of chlorinated phenols by strain DCMB5

Chlorophenol added[a]	Product(s) formed[b]		
	TeCP	TrCPs	DCPs
PCP	2,3,4,6^t > 2,3,5,6^t > 2,3,4,5^t	2,4,6 = 2,4,5 > 2,3,5^t	3,5 > 2,4/2,5[c]
2,3,4,6-TeCP		2,4,6 = 2,4,5	2,4
2,3,5,6-TeCP		2,3,5 ≫ 2,3,6	3,5 ≫ 2,5
2,3,4,5-TeCP		2,4,5 > 2,3,5	2,4/2,5[c]
2,4,6-TCP			2,4
2,3,5-TCP			3,5 ≫ 2,5
2,4,5-TCP			Traces of 2,5 and 2,4
2,3,4-TCP			2,4 > 3,4 (2:1)
2,3,6-TCP			2,5
3,4,5-TCP			3,5 > 3,4 (5:1)

[a] Initial concentrations were 50 (PCP and TCPs) and 30 (TeCPs) μM.
[b] Products are given in order of decreasing amount. Numbers in parentheses are ratios of products. A superior t indicates transient formation.
[c] Not separated by GC.

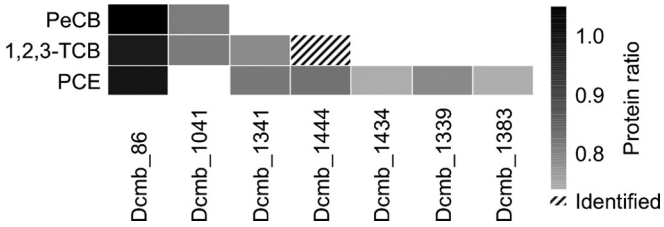

FIG 5 Detected RdhA proteins. The RdhA proteins are denoted by their locus tags, and the ratio to GroEL, as detected from the pellet fraction of samples grown with PeCB, 1,2,3-TCB, and PCE as electron acceptors, is shown. Proteins identified but not quantified are marked by a hatched box.

to TCE, but no further dechlorination products were detected. To test for growth with PCE as an electron acceptor, the cultures were transferred into fresh medium (10%, vol/vol). Four subsequent doses of PCE were dechlorinated to TCE within 42 days (see Fig. S4 in the supplemental material). The cell number increased from 8.7×10^6 ($\pm 8 \times 10^5$) to 1×10^8 ($\pm 2.1 \times 10^7$) ml^{-1}, resulting in a growth yield of 1.7×10^8 cells per μmol TCE formed. Dechlorination of α-, β- and γ-HCH and of a mixture of all HCH isomers was also analyzed. A slow decrease of the initial concentration of all isomers occurred in the live cultures as well as in the abiotic controls. The formation of penta- and tetrachlorocyclohexene was observed only for γ-HCH; however, both also accumulated in the abiotic controls. Formation of monochlorobenzene was not observed, indicating that strain DCMB5 does not dechlorinate the HCH isomers.

Proteome analyses of cells grown with different electron acceptors. Protein profiles during the cultivation with different electron acceptors were investigated and resulted in the identification of 475 (PeCB), 781 (1,2,3-TCB) and 912 (PCE) proteins, corresponding to between 32 and 62% of the 1,477 annotated protein-coding sequences of DCMB5. The low number of identified proteins from PeCB-grown cells can be explained as a result of losses during harvesting by coprecipitation of cells together with PeCB crystals. Notably, a common set of 425 proteins was identified under all three conditions, whereas 23, 59, and 182 were exclusively identified in PeCB-, 1,2,3-TCB-, and PCE-grown cells, respectively. From the pellet fractions, higher numbers of proteins were identified (1.4- to 2.7-fold higher counts) than from the supernatant fractions. An enrichment of membrane proteins (no enrichment with PeCB, but 2.1- and 2.3-fold more membrane proteins for 1,2,3-TCB and PCE, respectively) was also observed in the pellet fractions (see Table S1 in the supplemental material).

Despite 23 Rdh proteins being encoded in the genome of DCMB5, we were able to identify only seven during growth with at least one of the three chlorinated compounds (Fig. 5). They were represented in two or three replicates of the respective cultivation conditions and were, therefore, quantified and normalized in comparison with GroEL. Two additional Rdh proteins, namely, Dcmb_120 and Dcmb_184, were found in only one culture of PeCB- and PCE-grown cells, respectively, and, therefore, were not quantified (see Table S1 in the supplemental material). Dcmb_86, an ortholog of the chlorobenzene reductive dehalogenase CbrA in strain CBDB1, was present in the highest abundance (1.15 to 0.95 relative to GroEL) under all three cultivation conditions in both the pellet fraction (Fig. 5) as well as in the supernatant fraction (see Table S1 in the supplemental material). All other Rdh proteins were exclusively detected in the pellet fraction, and their abundance relative to that of GroEL was <1 (0.72 to 0.82). Interestingly, Dcmb_1041 was quantified in PeCB- and 1,2,3-TCB-grown cells but was not detected in PCE-grown cells (Fig. 5). The Rdh Dcmb_1341 was present in 1,2,3-TCB- and PCE-grown cells. Three Rdh proteins (Dcmb_1339, Dcmb_1434, and Dcmb_1383) were exclusively quantified in PCE-grown cells. Interestingly, some corresponding regulatory proteins, encoded in the vicinity of the respective *rdh* genes, were also represented; e.g., the MarR-type regulators Dcmb_1040 and Dcmb_1342 and the two-component system response regulators Dcmb_85, Dcmb_1386, and Dcmb_1440 were identified (see Table S1 in the supplemental material).

Surprisingly, proteins involved in the formation and function of type IV pili (TFP) (e.g., prepilins, TFP leader peptidase, secretion proteins, and assembly and twitching mobility protein) were identified after growth with all three electron acceptors, 1,2,3-TCB, PeCB, and PCE (see Table S1 in the supplemental material). This suggests that pilus formation is a general feature during growth of strain DCMB5.

DISCUSSION

Cell morphology and pilus formation. Cells of *D. mccartyi* are disc-like, with an average size of 0.3 to 1 μm in diameter, and are 0.1 to 0.2 μm thick (22–26). The cells of strain DCMB5 had a similar size. However, phase contrast images taken from fresh samples also showed slightly larger cells with diameters up to 1.4 and occasionally 2 μm. The various cell sizes encouraged us to study the size distribution during growth with 1,2,3-TCB. During exponential growth, cells were in general smaller, with the largest percentage of cells having diameters between 0.55 and 1 μm, whereas in the stationary phase, the majority of cells were 0.8 to 1.2 μm in size, and up to 15% of cells had diameters of 1.2 to 1.4 μm. The electron microscopic inspection of exponential-phase cultures documented the existence of pairs of unequal-sized cells, pointing to an irregular mode of binary cell division. The genome of strain DCMB5 encodes the divisome components, which are responsible for the constriction of the membrane (FtsZ and FtsA) and the translocation of the complete chromosome to the daughter cell (FtsK) (27, 28). However, genes encoding the classical divisome proteins for peptidoglycan synthesis as well as those involved in directing the Z ring to the middle of the cell are missing from the genome (27). This might suggest a rather primordial type of irregular cell division with the random formation of equal- and unequal-sized daughter cells. Unequal-sized cells are reminiscent of bud-like structures and have also been documented in microscopic images of other *D. mccartyi* strains, such as CBDB1, FL2, and MB (22, 23, 25).

The observation of filamentous structures, possibly pili, was surprising. Filamentous appendages had been observed earlier for other *D. mccartyi* strains (22, 29); however, they seemed to have a diameter greater than the 6 to 8 nm reported for type IV pili (TFP) (30, 31). Pili are very sensitive to mechanical damage and can be completely sheared from the cell surface by centrifugation at 8,000 × *g* (32). This might explain why they have been overlooked so far. In strain DCMB5, they were detected in the late exponential phase, in accordance with the reported growth-phase-dependent pilus expression of *Lactobacillus* (33). The genome of strain DCMB5 harbors a gene cluster (locus tags dcmb_1210 to dcmb_1221) that is highly conserved in all reported *D. mccartyi*

genomes and encodes TFP prepilins, the assembly and disassembly machinery, and the TFP-associated type II secretion system. The observed fibers attached to the cells appeared frequently in pairs and had an unusually small diameter of 3 nm, possibly constituting disaggregated single fibers of normally spiraling three-helix bundles (30, 31). Type IV pili are known to be involved in twitching motility, competence, and attachment (34). It is intriguing that in the presence of all three electron acceptors, 1,2,3-TCB, PeCB, and PCE, most of the TFP proteins were produced (see Table S1 in the supplemental material). In addition, the recently reported proteome of HCB-grown cells of strain CBDB1 also revealed the presence of TFP proteins, suggesting that pilus formation is a common property of *D. mccartyi* strains (10). The pili might contribute to the astonishing capacity of *D. mccartyi* strains to respire highly hydrophobic organochlorines like polychlorinated biphenyls (35), dibenzo-*p*-dioxins (5), and HCB (36), which are barely soluble and adsorb to solid material, so their consumption might require tight cell attachment.

Spectrum of organohalides used as electron acceptors and the possible involvement of specific Rdh proteins. Strain DCMB5 can grow by organohalide respiration using a broad spectrum of halogenated compounds as electron acceptors. The determined growth yield for 1,2,3-TCB of 10^7 cells per μmol chloride released was well in the range of that observed for other *D. mccartyi* strains (17, 23, 25, 37). However, DCMB5 particularly resembles *D. mccartyi* strain CBDB1 in its preference for haloaromatic compounds as electron acceptors. The observed dechlorination route of HCB and PeCB via both 1,2,3,5- and 1,2,4,5-TeCB to 1,2,4- and/or 1,3,5-TCB is identical to that of strain CBDB1; however, the latter strain can further productively dechlorinate 1,2,4-TCB, forming 1,3- and 1,4-DCB as final products (36, 38). Clearly, strain DCMB5 has a lower capacity to remove singly flanked chlorines. However, 1,2,3-TCB supported growth of strain DCMB5 and was dechlorinated to 1,3-DCB. The strains CBDB1 and DCMB5 are so far the only reported pure cultures using this compound as an electron acceptor, whereas *D. mccartyi* strains 195 and BTF08 and "*Dehalobium chlorocoercia*" DF-1 reductively dechlorinate only more highly chlorinated chlorobenzenes (20, 39, 40). The ability of strains CBDB1 and DCMB5 to dechlorinate chlorobenzenes might at least in part be attributed to the property of a chlorobenzene-reductive dehalogenase. CbrA has been gel purified from strain CBDB1 and was shown to dechlorinate 1,2,3-TCB and 1,2,3,4-TeCB (41). It possibly also plays a role in the dechlorination of HCB, because it constituted one of the most dominant Rdh proteins in the respective proteome (42). Strain DCMB5 possesses the only known ortholog of CbrA, Dcmb_86 (99% amino acid sequence identity), and its involvement in the dechlorination of 1,2,3-TCB and PeCB is suggested by its high abundance in the respective proteomes. The analysis of all further Rdh proteins detected in 1,2,3-TCB- and/or PeCB-grown cells in strain DCMB5 (Dcmb_1041, Dcmb_1341, and Dcmb_1444) (Fig. 5) revealed not only that the three Rdh proteins possessed orthologs in strain CBDB1 but also that these orthologs were identified with a high protein coverage in the proteome of HCB-grown cells of CBDB1 (10). Here we could demonstrate that formation of Dcmb_1041 (CBDB1 ortholog CbdbA1092) is dependent on the presence of PeCB or 1,2,3-TCB, whereas it was not detected in PCE-grown cells of the first and second transfer. When the higher proteome coverage of PCE-grown cells is taken into consideration (see Fig. S5 in the supplemental material), this clearly indicates that the formation of Dcmb_1041 was specific for the chlorobenzenes. Dcmb_1341 and Dcmb_1444 (respective CBDB1 orthologs CbdbA1455 and CbdbA1638) were identified in the proteomes of 1,2,3-TCB- as well as PCE-grown cells and, thus, seem not to be specifically induced.

Reductive dehalogenation of PCDD/Fs has been reported for different microcosms and enrichment cultures obtained from dioxin-contaminated fresh water or estuarine sediments worldwide (for a review, see reference 43). Only three pure cultures, all belonging to *D. mccartyi*, have been described to be capable of dechlorinating PCDD/F. Strain 195 is able to dechlorinate 1,2,3,7,8-pentachlorodibenzo-*p*-dioxin (PeCDD) and 1,2,3,4,7,8-hexachlorodibenzofuran almost exclusively at lateral positions, leading to tri- and tetrachlorinated final products, respectively (44, 45), and thus avoid the formation of the most toxic 2,3,7,8-substituted congeners. Accordingly, 1,2,3,4-TeCDD was dechlorinated to 1,2,4-TrCDD, and dechlorination stopped at the level of 1,3-DCDD. However, strain 195 did not grow on 1,2,3,4-TeCDD as the sole electron acceptor (44). The congeners 2,3,7,8-TeCDD and 2,3-DCDD were not dechlorinated (39). Strain CBDB1 also dechlorinated 1,2,3,7,8-PeCDD, forming the notorious 2,3,7,8-TeCDD as an intermediate, which was, however, further dechlorinated to DCDDs (5). In addition, CBDB1 can respire 1,2,3- and 1,2,4-TrCDD, in both cases leading to 2-MCDD as the final dechlorination product. Strain DCMB5 was capable of dechlorinating seven of the nine tested dibenzo-*p*-dioxin congeners with a remarkably short lag time after transfer from the 1,2,3-TCB-grown preculture (see Table S2 in the supplemental material). Growth of strain DCMB5 in enrichment cultures with 1,2,4- and 1,2,3-TrCDD was previously shown by the increasing copy number of its 16S rRNA gene (46). Here we demonstrated that the cell number of the isolated strain DCMB5 increased with 1,2,3,4-TeCDD and 1,2,4-TrCDD, indicating that these are electron acceptors in organohalide respiration. The main dechlorination routes resembled those of CBDB1, with peripheral chlorines (positions 1 and 4) consistently removed. Whereas strain CBDB1 can additionally remove one of two lateral chlorines, strain DCMB5 cannot, resulting in the accumulation of 2,3-DCDD as the main product during dechlorination of 1,2,3,4-TeCDD or 1,2,3-TCDD. Lateral dechlorination occurred only as a minor side reaction, leading to small amounts of 1,3-DCDD. However, the observation that trace amounts of 1-MCDD were formed from 1,2,4-TrCDD indicated that another side reaction exists for the removal of the chlorine in position 2 from 1,2,4-TrCDD. The resulting 1,4-DCDD was accessible to a complete peripheral dechlorination to the nonchlorinated dibenzo-*p*-dioxin. It was exclusively formed from 1,4-DCDD and 1-MCDD in strain DCMB5. Interestingly, 1,4-DCDD and 1-MCDD were observed as final dechlorination products in sediment microcosms dechlorinating 1,2,3,4-TeCDD (47), and 1-MCDD was formed in PCDD/F-dechlorinating cultures (48). However, the formation of dibenzo-*p*-dioxin has not yet, to our knowledge, been reported. This capability might provide the missing link in the reported total reduction of all PCDD/F congeners in *Dehalococcoides*-containing sediment microcosms, in which monochlorinated congeners appeared as dechlorination products and catechol and salicylic acid as products of a likely subsequent oxidative dibenzo-*p*-dioxin degradation (49).

Strain DCMB5 is also capable of dechlorinating pentachlorophenol, all three tetrachlorophenols, and six trichlorophenols. Growth with chlorophenols as electron acceptors was shown for

2,3,6-TCP as an example. However, the strain is unable to dechlorinate 2,3-DCP or the other DCPs formed during the dechlorination of higher chlorinated phenols. This is a remarkable difference from both *D. mccartyi* strains CBDB1 and 195 (17). In strain 195, the ability to dechlorinate 2,3-DCP was assigned to the bifunctional PCE/2,3-DCP dehalogenase PceA (50), an ortholog of which (CbdbA1588; 94% amino acid sequence identity) is also encoded in the genome of strain CBDB1 and was also formed as the most abundant Rdh in 2,3-DCP-grown cells (9). An ortholog of this Rdh is missing in strain DCMB5, and its catalytic capability obviously cannot be adopted by another Rdh. Despite the lack of a PceA ortholog, strain DCMB5 was surprisingly able to dechlorinate PCE to TCE, using it as an electron acceptor with a growth yield comparable to that obtained with *D. mccartyi* strain BTF08 grown on chlorinated ethenes (20). TCE, *cis*- and *trans*-DCE, and VC are not dechlorinated by strain DCMB5 and, correspondingly, genes encoding the classical TCE and vinyl chloride reductases (TceA and VcrA/BvcA) are missing in its genome. Notably, a close ortholog of *mbrA* (GenBank accession no. ADF96893.1), which was assigned a function in the dechlorination of PCE to *trans*-DCE in strain MB (51), is present in strain DCMB5 (dcmb_81). However, dcmb_81 was not detected on the protein level, either in PCE- or in chlorobenzene-grown cells, and therefore is likely not involved in dechlorination of PCE to TCE or in chlorobenzene dechlorination steps. It was surprising that Dcmb_86 was again the most abundant Rdh even after two transfers with PCE. Whether Dcmb_86 has a role in the dechlorination of PCE to TCE can only be determined by biochemical studies. So far it cannot be ruled out that unknown long-term regulatory effects keep its formation at a high level over consecutive transfers in the absence of the inducing compound, as has been reported for PceA in *Sulfurospirillum multivorans* (52). Two further Rdh proteins, Dcmb_1341 and Dcmb_1444, were indicated in the proteomes of the first and second transfer with PCE; however, the expression seems less substrate specific, because they were also detected in at least some replicates of 1,2,3-TCB-grown cells (Fig. 5; also, see Table S1 in the supplemental material). The dcmb_1444 ortholog DET1545 in strain 195 was shown to be transcriptionally upregulated in the stationary phase, also suggesting a substrate-independent induction (53). Furthermore, three Rdh proteins, Dcmb_1434, Dcmb_1339, and Dcmb_1383, were detected only in the presence of PCE. Notably, Dcmb_1434 and Dcmb_1339 were detected in cells from the first and second transfers (see Table S1 in the supplemental material). No orthologs have so far been reported for Dcmb_1434, and therefore, it is tempting to speculate that it might be specifically involved in the observed one-step dechlorination of PCE to TCE. However, the lower proteome coverage of 1,2,3-TCB- and PeCB-grown cells (see Fig. S5 in the supplemental material) might have so far prevented its detection under these conditions.

In conclusion, this study describes the growth of strain DCMB5 by organohalide respiration with a broad range of chlorinated compounds, possibly reflecting its adaptation to the diversity of pollutants at the site from which it was isolated. Strain DCMB5 is a second *D. mccartyi* strain, in addition to CBDB1, with a particularly high capacity to respire halogenated aromatic compounds, which, at least in part, might be facilitated by the activity of a CbrA ortholog. The intriguing ability of strain DCMB5 to completely dehalogenate chlorinated dibenzo-*p*-dioxins further expands the bioremediation potential of the genus *Dehalococcoides*. In addition, the unusual dechlorination of PCE to TCE in the absence of any of the reported reductive dehalogenases involved in the dehalogenation of chloroaliphatics supports the existence of "mosaics" of functionally redundant Rdh proteins enabling *D. mccartyi* populations to adapt rapidly to emergent halogenated electron acceptors.

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